EXCITEMENT IN VESICANT RESEARCH--YESTERDAY, TODAY AND TOMORROW

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ABSTRACT

The presentation focuses on the biochemical and cellular mechanisms that may be responsible for the development of the acute cutaneous sulfur mustard (HD) injury and their exploitation for establishing rational approaches for therapeutic intervention. Relevant background information identifies the known, toxicologically important chemical reactions of HD with cellular targets and describes the pathological events that lead to vesication. The penultimate event in the formation of large subepidermal blisters appears to be the premature, massive, and almost concurrent death of basal epidermal keratinocytes with release of injury-producing proteases and inflammatory mediators. The genotoxicity of HD--the major thrust of HD research for more than 40 years, and one that has made major contributions to our knowledge of molecular genetics--is briefly described. Evidence suggests, however, that a potent genotoxicity of HD does not play a causal role in the acute cutaneous HD injury. Tissue injury requires higher doses than does genotoxicity, takes less time to develop, and is not dependent on DNA cross-links. Next, the currently proposed hypotheses for HD cytotoxicity are described. Several papers presented at this conference were shown to support, partially, the poly(ADP-ribose) polymerase (PADPRP) hypothesis and two papers were consistent with the thiol-Ca^{2+} hypothesis. While the PADPRP hypothesis

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appeared to be upheld in HD-treated human peripheral blood lymphocytes (PBL), it was insufficient to explain the cytotoxicity in HD-treated human epidermal keratinocytes (HEK). The author suggests, however, that both cell types may be relevant models for the HD injury, the HEK responses representing those of basal keratinocytes in the acute injury phase, and the PBL responses mirroring those of resting epidermal stem cells preceding the healing phase. After identifying existing knowledge gaps, the author discusses two courses that future vesicant research might take. First and foremost is the continuation of current efforts which combine basic research studies with an intelligent screening program until effective medical antidotes against HD can be identified, evaluated, developed, and fielded. Second is a novel and speculative concept--namely, that a common nonspecific vesicant antidote may be identified which, when combined with agent-specific antidotes, could provide enhanced protection against military vesicants. The concept is analogous to the use of atropine and oxime in the treatment of poisoning by nerve agents. Calmodulin antagonists, which were reported to protect against thermal burns, frostbite, and other selected skin injuries, are discussed as potential common vesicant antidotes.

Colonel Hurst, Dr. Smith, distinguished panel, colleagues from here and abroad, and my many friends in the audience. I am delighted and honored to have been asked to deliver the keynote address on vesicants. I guess that my selection for this task was predicated on my many years of experience in mustard research. However, I want to caution you: "Experience," says Oscar Wilde, "is the name everyone gives to his mistakes." Believe me, I certainly have lots of experience. I will point out some of the mistakes that I know about as I go along. Most of them will probably fall into the category of TTUs, which is a term I learned form Henry Meier, and which stands for "True, True, Unrelated." TTU refers to research that is interesting and important, but is unlikely to provide meaningful answers for the problems to be solved. Mistakes of the TTU type usually are due to ignorance of basic underlying theory, wrong assumptions, misinterpretation of results, or any combination of these. One example of TTU research in which I was involved for many years was due to the incorrect presumption that the acute cutaneous sulfur mustard (HD) injury is caused by the inhibition of DNA synthesis and cell division--the most sensitive biological effect of this bifunctional alkylating agent. More about this later.

Today, I want to talk about the mechanisms that may be responsible for acute, vesicant-induced, incapacitating injuries to the skin, especially those produced by HD. Although HD also damages lung, eye, bone marrow, and the intestinal tract, more is known about HD injuries to the skin, and it is probable that knowledge of pathophysiological processes and successful antidotal approaches for skin exposures would have relevance for exposure of other tissues. In the latter part of my presentation, I will allude to the cutaneous effects of other military vesicants and will present a recently conceived rationale for a common vesicant antidote.[1]

Before giving you a road map for my address, I want to recognize the contributions that have been made in areas of vesicant research not covered in my talk: topical protectants, decontamination, detoxification, pretreatment, medical management, detection, diagnosis, monitoring, models,

decision-tree networks, screening of candidate drugs, etc. Interest in all of these areas has grown enormously over the past decade, demonstrated by many reports on these topics at this conference.

I also would like to recognize the many outstanding contributions to our understanding of vesicant injury mechanisms that have been made both here and abroad by a group of excellent and dedicated scientists. It is especially gratifying to see so many of our foreign colleagues at this meeting. WELCOME. I should point out that until the early to mid-1980s, the U.S. had sole responsibility among the NATO nations for conducting the basic studies on HD injury mechanisms. However, until its resurrection during the past decade, even the U.S. effort on vesicants was very limited. Indeed, from 1954, when I first joined the staff of the Biomedical Laboratory at Edgewood as a First Lieutenant, until around 1981, there were only a handful of intra- and extramural mustard scientists who successfully thwarted being washed away by the tide of research on nerve agents, which were perceived to pose a greater threat than were vesicants. All of this changed dramatically when Saddam Hussein decided to use HD against the Iranians and his own Kurdish population in the 1980s and threatened to employ this vesicant against Israel and the allied forces during the recent Gulf War. Hopefully, this new and concerted effort, using newly emerging concepts and state-of-the-art technology, will identify and exploit the underlying mechanisms leading to acute vesicant injuries and lead to effective treatment of these injuries.

The current presentation is entitled "Excitement in Vesicant Research: Yesterday, Today, and Tomorrow." The topics covered will not necessarily be in chronological order but they will reflect the evolution of ideas and concepts. I put the emphasis on Excitement because I often have good ideas at night and have to get out of bed to write them down, lest I forget. However, I want to tell you that the history of HD research was also replete with plenty of disillusionment. Thus, in reviewing the World War II research on the mechanisms of HD-induced skin injury, Renshaw gloomily concluded: "The bulk of the chemical evidence gives little hope that removal (of HD from critical targets with which it has reacted) can be effected by procedures which in themselves would not be highly injurious...." He goes on to say, "nor do there appear to be favorable clues suggesting a method of treatment based on exerting an action on tissues which would enable them to ward off or overcome the effects of H or its products."[2]

Perhaps Renshaw was right, but we HD researchers are a hardy bunch; we never give up. We desperately wanted to succeed. Spurred on by the excitement generated by the discovery of the double helical structure of DNA and the emergence of molecular genetics, we realized that HD, the first chemical shown to possess radiomimetic properties, could be a powerful tool for elucidating mechanisms of DNA replication, mitosis, cell division, mutagenesis, and carcinogenesis. The hunch was correct, and for the better part of four decades, many HD researchers made great contributions in these areas (recently reviewed by Fox and Scott,[3] and Papirmeister *et al.*[4]).

The excitement of HD research continued when we began to consider biochemical mechanisms of cell death, a subject about which very little was know until the mid-1970s. This new line of inquiry appeared to be especially relevant to the acute cutaneous HD injury, since massive basal cell death is closely associated with toxic mechanisms that emerge at vesicating doses. Research on both programmed (apoptotic) and induced cell death processes continued to accelerate over the past 15 years, and once again, HD contributed greatly to our knowledge of underlying mechanisms. Exploitation of this knowledge may provide the rational approach needed for therapeutic intervention.

To orient the audience, the presentation on Biochemical Mechanisms in the Acute Cutaneous Mustard Injury will be divided into three major headings and several subheadings:

YESTERDAY: WHERE ARE WE COMING FROM?

- Reactions of HD with cellular constituents.
  What are the biologically important targets?
- The acute cutaneous HD injury.
  What pathological changes lead to vesication?
- The genotoxicity of HD--A 40-year interlude.
  Are genotoxic events responsible for acute skin injury?

TODAY: WHERE ARE WE NOW?

- Current biochemical hypotheses for HD cytotoxicity and acute skin injury.
- What has been validated and what are the existing knowledge gaps?
- Personal reflections on the injury mechanism.
  Should we put all of our eggs in one basket?

TOMORROW: WHERE ARE WE GOING?

- Awareness of emerging concepts.
- Validation or rejection of old hypotheses.
- Prospects for therapeutic intervention. Can agent-specific and/or universal vesicant antidotes be developed?

Reactions of HD with Cellular Constituents

HD is a ubiquitous bifunctional alkylating agent capable of reacting with a large number of nucleophilic cellular constituents, including both low-molecular-weight compounds and the macromolecular DNA, RNA, and proteins.[4] The biologically important reactions are the bifunctional (cross-linking) and monofunctional alkylations of DNA bases--reactions that produce genotoxic effects at low doses of HD and can inhibit transcription and energy metabolism at high doses of HD.[3,4] Because of their smaller size, RNA or protein (enzyme) alkylations are likely to have little effect at toxicologically relevant doses. However, HD-alkylated proteins can be potent antigens and sensitize to a subsequent HD exposure.

Biologically important reactions of HD with low-molecular-weight compounds are likely to be confined to those with high affinities, such as glutathione (GSH). Since GSH defends cells against reactive oxidants, depletion of GSH by high doses of HD could result in cell death. Consistent with this view is the current finding by Gross and Smith that boosting the cellular GSH content by increasing its synthesis provides protection against HD cytotoxicity.[5] Reactions of HD with membrane phospholipids have not been reported.

The Acute Cutaneous HD Injury

The severity of the cutaneous HD injury is dose-dependent and, more particularly, directly related to the amount of HD fixed (alkylated) in skin.[2] Visible pathology, however, develops only after an asymptomatic latent period, the length of which decreases but is still considerable at vesicating and even necrotizing doses. Starting concurrently with an erythematous stage, mild to moderate edema begins at 3 to 6 hours and persists throughout the period of peak erythema and, in the case of higher doses, throughout vesicle formation.[2,4] With vesicating doses of HD, blister formation begins at about 16 hours after exposure, apparently as a result of basal cell destruction. The subepidermal cleavage plane is between the basal cell plasma membrane and the basement membrane. Ultrastructural studies of human skin grafted onto nude mice demonstrated disruptions of the basal cell plasma membrane as early as 12 hours after exposure, before blister formation. These disruptions become more extensive at 24 hours and are accompanied by splitting of hemidesmosomes from the basal lamina. The separated epidermal-dermal junction shows the broken fragments of anchoring filaments dangling from the roof of the blister cavity and a denuded but intact basal lamina on the floor of the blister.[6] In humans, the coalescence of small blisters gives rise to large bullae.

The take-home messages from the pathology of the acute cutaneous HD injury are: (a) The severity of the injury depends on the alkylation level in skin, (b) visible injury develops after an asymptomatic latent period, (c) massive death of basal cells immediately precedes or accompanies vesication, (d) acute skin injury develops at a time much earlier than that expected from genotoxic effects, and (e) epidermal-dermal separation and blister formation may involve the splitting of anchoring filaments by protease released from moribund or dead cells. Several reports being presented at this conference not only support the validity of these take-home messages, but also add greatly to our knowledge of the pathogenesis of the HD injury.[7-13]

The Genotoxicity of HD--A 40-Year Interlude

The genotoxicity of HD represents one of the loves of my life, having spent approximately three decades in this field. I am proud to have been able to work with the many pioneering scientists who were engaged in research on the effects of HD and other alkylating agents on DNA and DNA-related issues. HD research has contributed greatly to our understanding of

molecular genetics and cell biology. Among the important areas we learned about are mechanisms of normal, semiconservative DNA replication, cell-cycle traverse, DNA repair, mutagenesis and carcinogenesis. More recently, we also have been concerned with the metabolic effects of extensive DNA damage, which is a high-dose, nongenotoxic effect of HD with cytotoxic consequences.

By virtue of its ability to cross-link the complementary strands of DNA, low doses of HD can inhibit cell division--a property which has been extensively exploited for developing a variety of HD analogs for use in cancer chemotherapy. The formation of interstrand cross-links and the very large size of DNA renders this molecule the most functionally sensitive target of HD in cells. Transcription, translation, and enzyme catalysis--cellular activities that are dependent on biological entities of much lower molecular size than chromosomal DNA--are much less sensitive to HD. Thus cells that are prevented from synthesizing DNA by low doses of HD continue to generate energy and synthesize DNA and protein. As a result of such unbalanced metabolism, cells may enlarge, differentiate, or be induced to synthesize high levels of certain proteins. While some of these induced proteins may protect cells (e.g., metallothioneine, repair enzymes), others may hasten cell death (e.g., protease, phospholipase, and nucleases). Another low-dose genotoxic effect of HD on somatic cells is mutagenesis, which can be caused either by replication errors or by misrepair; these mutations may contribute to the long-term health hazard of HD.[14] Several recent reviews and references cited therein provide further details on the genotoxicity of HD.[2,4,14]

Why do I now consider genotoxicity research to have been a 40-year interlude? In my opinion, inordinate emphasis may have been placed on the highly potent genotoxicity of HD because of the presumption that knowledge of the most HD-sensitive cellular function (i.e., DNA replication and normal cell division) would *a priori* lead to the elucidation of the acute injury mechanism. Such undue emphasis on low-dose effects of HD may have precluded adequate consideration of other injury mechanisms that emerge at higher doses. It has been known since the 1940s that the severity of the skin injury produced is directly related to the amount of HD fixed in the tissue. Careful examination of these and other data reveals that vesication and acute tissue injury occur only at fixation levels much higher that those needed to produce genotoxic effects. Tissue-injurant doses, however, are consistent with levels of alkylation which would cause metabolic and other cellular disturbances, such as inhibition of glucose metabolism[15] and elevation of intracellular calcium (Ca_i).[16] Furthermore, cross-alkylation is not a requirement for producing acute injury since, at comparable levels of alkylation, monofunctional sulfur mustards have been shown to be equally effective as vesicants. Also supporting this view is the finding that tissue injury does not develop when low, therapeutically effective doses of HD are used to control the hyperproliferation of psoriatic keratinocytes. Finally, it is probable that manifestations of low-dose cellular effects such as those that may result from unbalanced growth--irreversible cell enlargement or induction of catabolic enzymes--would take much longer to develop than would the acute HD injury. These considerations, in conjunction with a new awareness of emerging concepts of cell death

processes, have led investigators to consider nongenotoxic mechanisms in HD vesication.

Current Theories of Sulfur Mustard Cytotoxicity

The excitement in HD research continued in the early 1980s when we began to consider mechanisms of cell death induced by toxicants. Interest in cell death processes began around this time, and continued to mushroom over the next decade. Today, the topics of programmed (apoptotic) and the necrotic cell death mechanisms are major research thrusts. Presently, I will present a synopsis of essential elements of three biochemical hypotheses that have been proposed recently to account for HD cytotoxicity. Next, I will analyze the merits of each hypothesis, based largely on the data presented at this conference. Finally, I will identify important knowledge gaps. (For an in-depth discussion of HD cytotoxicity and injury-producing mechanisms, see Papirmeister *et. al.*[4]).

The Poly(Adenosine Diphosphoribose) Polymerase (PADPRP) Hypothesis. The PADPRP hypothesis, conceived in 1981 and published in 1985,[17] proposes a biochemical pathway whereby initial alkylation of DNA by HD promotes secondary DNA damages that serve as a primary cause of alterations of energy metabolism leading to cell death and pathology. The fundamental link is the activation of the chromosomal NAD^+-depleting enzyme, PADPRP, by DNA breaks sustained at apurinic sites. The reaction is followed by depletion of NAD^+, inhibition of glycolysis, and loss of cellular energy, and can have several other lethal consequences, such as the induction and secretion of increased levels of proteases and other degradative enzymes that are responsible for tissue injury.

The Thiol-Ca^{2+} Hypothesis. Whitfield suggested in 1987[18] that the cytotoxicity of HD may be explained by a mechanism originally proposed in 1985 by Orrenius to account for oxidant toxicity in hepatocytes. The thiol-Ca^{2+} hypothesis has since been expanded considerably,[19] and is now considered by many researchers to be generally applicable to the toxicity of a number of reactive electrophiles, perhaps including HD. The thiol-Ca^{2+} hypothesis proposes that by alkylating glutathione (GSH), HD removes one of the major cellular defense mechanisms against electrophilic compounds and oxidants. Once GSH is depleted, electrophiles such as HD or endogenously generated reactive oxygen species eventually inactivate critical sulfhydryl proteins involved in Ca^{2+} homeostasis and/or modify cytoskeletal elements. The subsequent inability of cells to maintain a low intracellular $Ca^{2+}(Ca_i)$ concentration causes activation of catabolic processes leading to cell damage and death. The thiol-Ca^{2+} hypothesis differs from the PADPRP hypothesis in that it proposes different initiating targets for HD cytotoxicity.

The Lipid Peroxidation Hypothesis. The lipid peroxidation hypothesis proposes that HD-induced depletion of GSH results in an increase in the levels of endogenously produced oxygen radicals that initiate lipid peroxidation, leading to membrane damage and cell death. The lipid

peroxidation hypothesis differs from the thiol-Ca^{2+} hypothesis in that it suggests other critical targets for endogenously produced oxidants.

The possibility must be entertained that several of the proposed pathways of HD cytotoxcity interact with one another. While initiating reactions can differ, subsequent sequences may overlap. This possibility reflects the fact that cell death caused by any initiating mechanism may proceed via a common terminal pathway.[20] Also, it must be recognized that due to the multiple molecular targets of HD, more than one potentially lethal mechanism may be operating concurrently and that interference with one pathway may be insufficient to prevent cell death (although death may be delayed). More about this later.

The findings reported at this conference either supported, partially supported, did not support, or did not address the aforementioned proposed mechanisms of HD cytotoxicity. The only study that almost fully validates the PADPRP hypothesis is that by Meier and Kelly,[21] who demonstrated that PADPRP inhibitors prevent the HD-induced losses of ATP, NAD^+, and viability in human peripheral blood lymphocytes (PBL); however, their observation that the decline of ATP precedes that of NAD^+ requires explanation. Support for some early steps of the PADPRP hypothesis is provided by the results of studies by Clark and Smith,[22] which showed that HD treatment of HeLa cells produces a rapid stimulation of PADPRP activity and is followed two hours later by a decline in NAD^+ levels. Only partial suport for the PADPRP hypothesis was obtained in several other studies: Smith *et al.*[23] noted that while niacinamide (a PADPRP inhibitor) prevents metabolic death of HD-exposed epithelial cells, it inhibits DNA repair, and may cause (delayed?) cytotoxicity; Cowan *et al.*[12] observed that although niacinamide attenuates HD-induced increases in protease activity *in vitro* and *in vivo*, it does not eliminate them, suggesting that pathways other than the PADPRP-initiated sequence can contribute to the enhancement of protease activity; Yourick *et al.*[8] noted that while niacinamide reduces the incidence of HD-induced microvesiculation in hairless guinea pig skin, the prediction of the PADPRP hypothesis that the loss of NAD^+ precedes tissue injury was not upheld; and finally, Martens and Smith[15] demonstrated that whereas HD treatment of human epidermal keratinocytes (HEK) produces a dose-dependent depletion of NAD^+ and inhibition of glucose metabolism, preceding cell death, the prediction of the PADPRP hypothesis that niacinamide would prevent the inhibition of glycolysis was not upheld, suggesting that in HEK, other energy-depleting mechanisms may be involved in HD cytotoxicity.

In partial support of the thiol-Ca^{2+} hypothesis were the observations by Ray *et al.*[16] that HD treatment of neuroblastoma cells or of HEK causes partial depletion of GSH, raises the level of Ca_i, and as previously reported by these authors, stimulates phospholipase A_2--processes that precede and ultimately lead to membrane damage and cell death. These results of Ray *et al.*, however, do not exclude the participation of other toxicity pathways for elevating Ca_i. Finally, tentative support of the thiol-Ca^{2+} hypothesis or the lipid peroxidation hypothesis is the finding by Gross and Smith[5] that, by increasing cellular GSH levels, human PBL are rendered more resistant to HD. However, the possible dose-reducing effects (detoxification) of increased GSH content must be evaluated.

Based on these findings, it appears that the PADPRP hypothesis is sufficient to account for HD cytoxicity in resting lymphocytes, but the hypothesis does not fully explain either the HD cytotoxicity in HEK or the acute skin injurant effects of HD. Other toxicity pathways are likely to contribute to HD-induced pathology.

Personal Perspectives on the Cutaneous HD-Injury

It is natural to inquire whether the resting human PBL or the cycling HEK represents a more realistic model for the cutaneous HD injury. My personal opinion is that both cell types are relevant, the HEK model being representative of the acute injury phase and the PBL model being representative of the healing phase of the injury. This opinion is based on the following notions. The acute cutaneous HD injury is preceded or accompanied by premature death of most of the cells of the basal layer of the epidermis. It is known that 95% of these basal keratinocytes are normally programmed to undergo several rounds of cell division before terminally differentiating and giving rise to the inviable stratum corneum. The immediate cause of the acute injury appears to be the premature, sudden, and massive release of destructive enzymes and mediators of inflammation.

The healing phase, on the other hand, involves the replacement of the epidermis and is initiated by stimulation of quiescent epidermal stem cells.[24] Most of the stem cells--approximately 1-5% of the total basal keratinocytes--are in the resting phase of the cell cycle (G0) but can be stimulated to divide as required for regulation of cell growth in a self-renewing tissue such as the epidermis. The stimulated stem cells are also responsible for replacing the epidermal cells during the healing phase of skin injuries. Recently, a population of homogeneously small keratinocytes with properties expected of stem cells was isolated from newborn rat skin.[25] These cells have a large nucleus, contain little cytoplasm, RNA, and calcium binding proteins, and are shown (by flow cytometry) to be in the G0 stage of the cell cycle. They do not actively proliferate *in vivo* but could be induced to divide and differentiate *in vitro*. Many of these properties of epidermal stem cells are similar to those of human PBL. It would be important to know if quiescent epidermal stem cells can be protected from HD cytotoxicity by PADPRP inhibitors and whether their potential for normal cell division can be maintained by efficient repair of DNA cross-links. Inferences from old studies in rabbits are consistent with a high capability of healing of HD-injured skin.

Knowledge Gaps

- How important are DNA repair and cell cycle traverse in basal keratinocytes, cells that are programmed to undergo terminal differentiation leading to the formation of the nonviable stratum corneum?

- Why do PADPRP inhibitors prevent losses of NAD^+, ATP, and viability in HD-treated human peripheral blood lymphocytes (PBL) but fail to prevent

HD-induced cytotoxicity in human epidermal keratinocytes (HEK) or HD-induced acute skin injury?

- Is HD-induced glycolysis inhibition and energy depletion in HEK accomplished by pathways other than the PADPRP-mediated NAD^+ loss?
- What mechanism(s) is(are) responsible for increasing and maintaining high levels of intracellular calcium in HD-treated cells?
- Are the increases in the activities of catabolic enzymes (e.g., proteases, phospholipases, endonucleases) in HD-exposed cells and skin due to induction of new enzymes or stimulation of existing enzymes?
- Does HD-induced inhibition of protein synthesis (either through loss of cellular energy or through inhibition of either transcription or translation) play a role in vesication in light of the known rapid turnover of endogenous protein inhibitors of catabolic enzymes?
- What contributions do apoptotic (programmed) and necrotic cell death processes make to the cytotoxic and acute skin injurant actions of HD?
- Is the protective action of glutathione against HD cytotoxicity due to the detoxification of HD, the protection of the cellular protein thiol status, or both?
- Do reactive oxygen species contribute to HD cytotoxicity?
- What is the role of inflammation in the development of the acute cutaneous HD injury?

Searching for Agent-Specific and Universal Vesicant Antidotes

One of the major goals of future medical chemical defense research on vesicants will continue to be the search for effective prophylactic and therapeutic countermeasures. The approach toward achieving this goal for HD exposures will continue involve the present combination of an intelligent screening program of candidate antidotes and basic research on injurant pathways with a view toward identifying new and more effective therapies. These are not mutually exclusive approaches but require awareness and close collaboration between scientists.

A model scheme which combines the two approaches was recently provided by Dr. Alan Feister, my coworker and a coauthor of our recent book on HD.[4] This scheme (see figure 9.1 in reference 4) is a composite of, and shows the interactions between, several of the hypothetical biochemical mechanisms of HD cytotoxicity, and identifies specific sites for potential therapeutic intervention. Nine major classes of drugs are indicated (each class being comprised of many subclasses): HD scavengers, DNA repair agents, NAD^+-level stabilizers, energy-level stabilizers, calcium-level stabilizers, autolytic enzyme inhibitors, antioxidants, cell-cycle regulators, and anti-inflammatory drugs. A number of candidate drugs, representing some but not

all of the classes, have already been examined both in screening programs and in basic research studies. Although progress has been made in identifying some compounds that appear efficacious in one model, the same compounds were sometimes found to be only partially effective, or even ineffective, in another model--leaving the question of what constitutes a relevant model unanswered. The problem is amply illustrated at this conference by the reported high therapeutic efficacy of the PADPRP inhibitor niacinamide in HD-treated lymphocytes and its lower therapeutic efficacy in HD-exposed HEK and skin. The difficulties in identifying effective HD antidotes can be traced to inherent differences in the screening model used, uncertainties regarding the injurant pathways involved, lack of potency and/or specificity of the drug selected for study, and the possibility that several independent or interacting pathways contribute to toxicity. To sort out all of these possibilities is a daunting task; however, while awaiting new developments, we can expect the present approaches for identifying HD antidotes to continue.

During the last several months I struggled with an even greater challenge. I was asked by the Army to explore the possiblity that a common vesicant antidote which would be effective against all military vesicants--HD, lewisite, phogene oxime, and T-2 mycotoxin--could be identified.* This challenge at first appeared insurmountable in view of the difficulties encountered with the most studied of the vesicants--HD. A ray of hope for meeting this challenge appeared, however, when I started to think about common antidotes against nerve agents. The first thing that is given to the nerve agent casualty is atropine, which prevents the life-threatening cholinergic crisis, a common manifestation of poisoning by all nerve agents. Atropine does not provide a therapy--it buys time. Therapy is achieved only when a critical level of the inhibited acetylcholinesterase has been restored (either by spontaneous or oxime-induced reactivation). And so it also may be with a common antidote against vesicants. What may be needed is to identify and block a converging but still reversible sequence that is common to all vesicants, and that probably involves a late step in the toxicity pathway.

A good late sequence to consider would be the increase in Ca_i, which is a common feature in cytotoxic sequences produced by different classes of compounds[20] and which was shown to be an important converging step in Dr. Feister's model of different proposed pathways of HD cytotoxicity. Moreover, since calmodulin mediates many of the cellular effects of Ca^{2+}--enzyme activation, microtubule disassembly, regulation of metabolic pathways, regulation of Ca^{2+} fluxes--the effects of calmodulin antagonists could be a good indicator of both mechanistic relevance and antidotal potential.

Recent evidence from studies of thermal injury suggests that calmodulin antagonists may be effective antivesicant compounds. In particular, Beitner *et al.*[28] have shown that CGS9343B, a novel, potent, and specific inhibitor of

*The current issue of Medical Chemical Defense' provides further details on the rationale for a common vesicant antidote.

calmodulin, is highly effective in treating skin injuries caused by exposure of 45% of the skin surface of anesthesized rats to boiling water. Injection or topical application of other calmodulin inhibitors has been reported to provide both prophylactic and therapeutic benefits in thermal burns by preventing or restoring the biochemical, physiological, and morphological changes.[27] These benefits include raising the depressed ATP levels and the reduced activities of 6-phosphogluconate dehydrogenase and hexokinase to normal control levels; preventing changes in the ultrastructural appearance of the skin; protecting the integrity of blood capillaries and their erythrocyte membrane; and reducing the hemoglobin content of burned skin.[26]

These findings prompted Beitner and co-workers[26] to propose a mechanism underlying the antidotal effects of calmodulin antagonists in thermal burns and in other selected injuries such as those caused by frostbite, bradykinin, and serotonin. Briefly, it is postulated that injured skin releases pharmacologically active chemicals such as bradykinin, serotonin, and histamine, which increase Ca_i that, in turn, binds to calmodulin to form the active Ca^{2+}-calmodulin complex. The active complex activates phospholipase A_2, and damages the membrane and causes leakage of enzymes. In addition, the Ca^{2+}-calmodulin complex activates glucose-1,6-bisphosphatase, leading to a decrease in glucose-1,6-bisphosphate, inhibition of glycolysis, and lowering of the ATP content. The reduced ATP levels further enhance Ca_i by inhibiting calcium-pumping ATPases, causing a mitochondrial calcium overload with further depression of ATP, exacerbation of membrane damage, enhanced leakage of enzymes, irreversible loss of Ca^{2+} homeostasis, and cell death, and culminating in typical burn pathology. Calmodulin antagonsists are proposed to block these destructive cycles by rendering the Ca^{2+}-calmodulin complex functionally inert and incapable of activating phospholipase A_2 and glucose-1,6-bisphosphatase.

A causal role for the Ca^{2+}-calmodulin second messenger system in the development of acute injuries by HD, lewisite, phosgene oxime, or T-2 mycotoxin is, at present, speculative. The following fragmentary bits of circumstantial evidence obtained with military vesicants, however, appear consistent with the Beitner model for thermal burns and its prevention by calmodulin antagonists: (a) The development of immediate or delayed pain and itching caused by exposure of skin to military vesicants is probably due to release of bradykinin, histamine, or other mediators. HD has been shown to cause release of histamine[10] and cytokines.[12] (b) An increase in intracellular Ca^{2+} and an activation of phospholipase A_2 leading to membrane damage have been demonstrated in several mammalian cell lines following exposure to HD.[18] (c) Levels of glucose-1,6-bisphosphatase following exposure to vesicants have not been measured, however, the ability of hexosediphosphate to reverse the HD-induced inhibition of glycolysis is consistent with activation of this enzyme.[4] (d) Leakage of various cellular enzymes from HD-exposed cells and skin has been demonstrated (e.g., lactic acid dehydrogenase, protease). The delayed loss of hexokinase from HD-exposed skin may also be caused by leakage from moribund or dead cells.

If the calmodulin hypothesis is validated for vesicant-induced injuries and a common antidote is identified, it is anticipated that the addition of a late-acting common antidote may be combined with and may complement the

individualized specific countermeasures that act at early stages of the pathogenic sequences. For example, the addition of a calmodulin antagonist to BAL or to niacinamide may greatly enhance the therapeutic efficacies of these agent-specific compounds against the injurant effects of L and HD, respectively. Such drug combinations might have additive or even synergistic antidotal properties. Moreover, by addressing both early and late pathogenic sequences, drug combinations may be effective over a longer period.

In conclusion, I would like to say that these are my personal views--some factual, some speculative--regarding the injurant mechanisms of vesicants and rationales for potential therapy. These views are not cast in concrete and are subject to change by substituting superior alternatives. I don't mind being proven wrong. My intent here is to open debate and keep the excitement in vesicant research alive until, after three quarters of a century of trying, we can finally come up with effective medical countermeasures.

References

[1] Papirmeister, B. (1993), *Med. Chem. Def. 6*,1. [2] Renshaw, B. (1946). In: *Chem. Warfare Agents and Related Chem. Problems*, Parts III-VI, Ch. 23, DTIC No. AD-234249. [3] Fox, M. and D. Scott (1980), *Mutat. Res. 75*, 131. [4] Papirmeister, B., A.J. Feister, S.I. Robinson, and R.D. Ford (1991). In: *Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*, CRC Press, Boca Raton, FL. [5] Gross, C.L., and W.J. Smith (1993), this volume. [6] Papirmeister, B., C.L. Gross, J.P. Petrali, and H.L. Meier (1984), *J. Toxicol. Cutaneous Ocul. Toxicol.* 3, 393. [7] Petrali, J.P., S.B. Oglesby, T.A. Hamilton, and K.R. Mills (1993), this volume. [8] Yourick, J.J., J.S. Dawson, C.D. Benton, M.E. Craig, and L.W. Mitcheltree (1993), this volume. [9] Monteiro-Riviere (1993), this volume. [10] Graham, J.S., M.A. Bryant, and E. H. Braue (1993), this volume. [11] Bernstein, I.A., L. Bernstam, Y.H. Yang, and F.L. Vaughan (1993), this volume. [12] Cowan, F.M., J.J. Yourick, C.G. Hurst, C.A. Broomfield, and W.J. Smith (1993), this volume. [13] Dannenberg, A.M., Jr., and J. Tauruta (1993), this volume. [14] Pechura, C.M., and D.P. Rall, editors (1993). In: *Veterans at Risk, the Health Effects of Mustard Gas and Lewisite*, Nat. Acad. Press, Washington, DC. [15] Martens, M.E., and W.J. Smith (1993), this volume. [16] Ray, R., J. Majerus, G.S. Munavalli, and J.P. Petrali (1993), this volume. [17] Papirmeister, B., C.L. Gross, H.L. Meier, J.P. Petrali, and J.B. Johnson (1985), *Fund. Appl. Toxicol. 5*, S134. [18] Whitfield, D. (1987), CDE, Porton, Wilts, U.K., Technical Note No. 840. [19] Orrenius, S., D.J. McConkey, and P. Nicotera (1988). In: *Oxy-Radicals in Molecular Biology and Pathology*. Alan R. Liss, Inc., NY. [20] Schanne, F.A.X. *et al.* (1979), *Science 206*, 700. [21] Meier, H.L., and S.A. Kelly (1993), this volume. [22] Clark, O.E. and W.J. Smith (1993) this volume. [23] Smith, W.J., K.M. Sanders, S.E. Ruddle, and C.L. Gross (1993), this volume. [24] Potten, C.S. (1986), *Br. J. Cancer 53*, Suppl. VII, 47. [25] Pavlovitch, J.H., M. Rizk-Rabin, P. Jaffray, H. Hoen, and M. Poot (1991), *Am. J. Physiol. 261 (Cell Physiol. 30)* [26] Beitner, R., M. Chen-Zion, and Y.B. Bassukevitz (1991), *Gen. Pharmacol. 22*,67. [27] Beitner, R., M. Chen-Zion,

Y. Sofer-Bassukevitz, Y. Oster, H. Ben-Porat, and H. Morgenstern (1989), *Gen. Pharmacol.* 20, 165.